

# Spectroscopic characterization of a novel $2 \times [4\text{Fe}-4\text{S}]$ ferredoxin isolated from *Desulfovibrio desulfuricans* ATCC 27774

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## Abstract

A novel iron–sulfur containing protein, a ferredoxin (Fd), was purified to homogeneity from the extract of *Desulfovibrio desulfuricans* American type culture collection (ATCC) 27774. The purified protein is a 13.4 kDa homodimer with a polypeptide chain of 60 amino acids residues, containing eight cysteines that coordinate two  $[4\text{Fe}-4\text{S}]$  clusters. The protein is shown to be air sensitive and cluster conversions take place. We structurally characterize a redox state that contains two  $[4\text{Fe}-4\text{S}]$  cores. 1D and 2D  $^1\text{H}$  NMR studies are reported on form containing the clusters in the oxidized state. Based on the nuclear Overhauser effect (NOE), relaxation measurements and comparison of the present data with the available spectra of the analogous 8Fe Fds, the cluster ligands were specifically assigned to the eight-cysteinyll residues.

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## 1. Introduction

Iron–sulfur (Fe–S) proteins are a class of proteins containing prosthetic groups composed of iron and sulfur atoms, known as iron–sulfur clusters [1–4].

Ferredoxins (Fd) are small and acidic iron–sulfur proteins that can be used as “model” compounds in the investigation of electronic, magnetic and structural properties of the iron–sulfur clusters in complex enzymes. They are classified according to the type of  $[\text{Fe}-\text{S}]$  aggregates. Three type of centers can be found:  $[2\text{Fe}-2\text{S}]$  centers originating from Fds isolated of plants and

animals, and  $[3\text{Fe}-4\text{S}]/[4\text{Fe}-4\text{S}]$  typically derived from bacteria. In sulfate reducing bacteria (SRB) four distinct types of Fds can be found based on the iron–sulfur cluster(s) content: 3Fe Fds containing one  $[3\text{Fe}-4\text{S}]$ ; 4Fe Fds containing one  $[4\text{Fe}-4\text{S}]$ ; 7Fe Fds containing a  $[3\text{Fe}-4\text{S}]$  and a  $[4\text{Fe}-4\text{S}]$ ; and 8Fe Fds containing two  $[4\text{Fe}-4\text{S}]$ . The cluster iron ions,  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$ , are tetrahedrally coordinate by sulfur atoms. Its binding to the polypeptide chain is made by a cysteic sulfur atom [5–9] or, sometimes, by an oxygen or nitrogen atom [10]. In the late years, spectroscopic techniques (X-ray crystallography, X-ray absorption, EPR, NMR, ENDOR, CD, MCD, Mössbauer, optical absorption and Raman resonance), have been of main importance in the biological characterization of the iron–sulfur centers, providing detailed description of their structures and electronic and magnetic properties.

Besides its important role in electron transfer reactions [1–4,11], iron–sulfur clusters are now known to participate in the activation of substrates [12–14], stabilization of radicals and structures [15,16], protec-

**Abbreviations:**  $^1\text{H}$  NMR, proton nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy; Fd, ferredoxin; *Dd*, *Desulfovibrio desulfuricans*; ATCC, American type culture collection.

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tion of proteins from enzyme attacks [17], storage of iron and sulfur [18], sensors of iron, dioxygen, superoxide ion ( $\text{O}_2^-$ ) and possibly nitric acid [19–23] and are involved in the expression of genes [24].

A new Fd has been isolated and purified from *Desulfovibrio desulfuricans* American type culture collection (ATCC) 27774 bacteria. The purified protein is quite unstable in the presence of oxygen. It is a dimer of 13.4 kDa molecular mass, that exhibits an optical spectrum typical of iron–sulfur proteins. The amino acid sequence composed of approximately 60 amino acids residues, includes eight cysteines, and show a great homology with the ones of other 7Fe and 8Fe Fds, namely the *Desulfomicrobium baculatum* Norway 4 Fd (DmbFd) [25], *Clostridium pasteurianum* ferredoxin (CpFd) [26] and *Pseudomonas nautica* ferredoxin (PaFd) [27]. Based on this homology, as well as in the solution structure of CpFd [28], the 3D crystal structures of *Peptococcus aerogenes* ferredoxin (PaFd) [29], *Clostridium acidurici* (Cau) [30] and DmbFd [25], together with the  $^1\text{H}$  1D and nuclear Overhauser enhanced spectra (NOESY 2D NMR) of Cp and Dd27 Fds, the clusters ligands were specifically assigned.

## 2. Experimental

### 2.1. Organism

*D. desulfuricans* ATCC 27774 was grown in a media described by Liu and Peck [31]. Cell free extract was suspended in Tris–HCl 10 mM, pH 7.6 buffer and cells lysed in cellular presson apparatus “French Press”, at 9000 Psi. The extract was centrifuged at  $19\,000 \times g$  for 30 min and then at  $18\,000 \times g$  for 75 min.

### 2.2. Protein purification

Due to oxygen sensitivity all the buffers were previously degassed and kept under continuous  $\text{N}_2$  flux when handled. Protein collecting was also done in previously degassed flasks and under continuous  $\text{N}_2$  flux. Cell-free extract was first applied onto a DEAE-52 column (35 by 3.5 cm) equilibrated in 10 mM Tris–HCl buffer (pH 7.6). Bound proteins were eluted with a 600 ml linear gradient of 10–400 mM Tris–HCl buffer (pH 7.6). The Fd containing fractions, eluted between 380 and 400 mM, were concentrated by Amicon YM-5 ultra filtration and equilibrated with 10 mM Tris–HCl buffer (pH 7.6). Fd fraction was then applied onto a pre-packed Source-Q XK-26 column coupled to a HiLoad 26/10 “Fast flow column”, both from Amersham Biosciences and previously equilibrated with 10 mM Tris–HCl buffer (pH 7.6). Bound proteins were eluted with a 400 ml non-linear gradient ( $3 \text{ ml min}^{-1}$ ) of 10–400 mM Tris–HCl buffer (pH 7.6). The Fd containing

fractions eluted at 400 mM were concentrated as previously described above. Fd was further purified by gel filtration on a pre-packed Superdex 75 HR 10/30 column coupled to a HiLoad 26/10 “Fast flow column”, both from Amersham Biosciences and previously equilibrated with 50 mM Phosphate buffer (pH 7.6) containing 150 mM NaCl. Fd fractions were judge to be pure according to the 17.5% polyacrylamide SDS Page electrophoreses gel. These fractions were concentrated as previously described and stored at  $-20^\circ\text{C}$  under  $\text{N}_2$  in 10 mM Tris–HCl (pH 7.6) buffer.

### 2.3. Spectroscopy

#### 2.3.1. Ultraviolet–visible

Ultraviolet–visible (UV–Vis) absorption spectra were measured at room temperature in the absence of oxygen using a Shimadzu UV-265 spectrophotometer and an anaerobic 1 cm quartz cell.

#### 2.3.2. Electron paramagnetic resonance

EPR spectra were carried out on a Bruker EMX 8/2.7 spectrometer equipped with a continuous flux cryostat from Oxford instruments. Samples were prepared with pure protein concentrated to 150  $\mu\text{M}$ , in 100 mM phosphate buffer, pH 8.0. Sample reduction was done by addition of sodium dithionite.

#### 2.3.3. Nuclear magnetic resonance

NMR spectra were recorded in a 400 MHz ARX Bruker spectrometer, equipped with a temperature unit control. Chemical shifts values were quoted in parts per million relative to 3-trimethylsilyl-(2,2,3,3- $^2\text{H}_4$ ) propionate.  $T_1$  values were determined by the inversion recovery method [32]. Phase-sensitive NOESY [33,34] spectra were recorded with short mixing times, 2–10 ms, with 1024 $t_2$  points and 256 $t_1$  blocks and 3000–4000 scans per block. Repetition times varied from 75 to 100 ms. Samples were prepared with pure protein, equilibrated with 100 mM Phosphate buffer, pH 8.0 and exchanged four times with 99.9%  $^2\text{H}_2\text{O}$ . Final protein concentration was 2 mM. Sample reduction was accomplished by adding directly to the degassed NMR tube (nitrogen atmosphere) aliquots of sodium dithionite prepared in  $^2\text{H}_2\text{O}$ , pH 8.0. Slow sample reoxidation was attained by introduction of controlled amounts of air, using gas-tight Hamilton syringes.

### 2.4. Molecular weight determination

The molecular weight of Dd27Fd was determined by gel filtration on pre-packed Superdex 75 HR 10/30 column coupled to a HiLoad 26/10 “Fast flow column”, both from Amersham Biosciences and previously equilibrated with 50 mM Phosphate buffer, pH 7.6, 150 mM NaCl. The following proteins were used as markers:

serum bovine albumin (67 kDa), ovalbumin (43 kDa), quimiotripsin (25 kDa), ribonuclease (13.7 kDa) and *Desulfovibrio gigas* Rubredoxin (6 kDa).

### 2.5. Protein and iron quantification

*Dd27Fd* was quantified using the BCA method (BCA protein assay reagent kit, Pierce), according to manufacturer specifications, using *P. nautica* Fd as standard. Iron quantification was done by a chemical method using 2,4,6-tripiridil-s-triazine (TPTZ), as described by Fisher and Price [35].

### 2.6. Amino acid sequence determination

Amino acid sequence determination was performed on an automatic sequencer (Applied Biosystem 477A), coupled to an amino acid analyzer (Applied Biosystem 120A) according to the experimental procedures described by Edman [36] and Laursen [37].

## 3. Results and discussion

The fact that the amount of protein isolated under aerobic conditions was merely residual, led to a change in the purification process to anaerobic conditions. The UV–Vis spectrum of pure native *Dd27Fd* presents absorption maxima at 275, 315 and 390 nm and a ratio  $\text{Abs}_{390\text{ nm}}/\text{Abs}_{275\text{ nm}} = 0.59$ . It was observed (data not shown) that the characteristic Fe–S charge transfer at 390 nm fades away after exposition of the protein to oxygen.

The polypeptide chain contains 60 amino acid residues, eight of them are cysteinyl residues: Cys11, 14, 17, 21, 41, 44, 47 and 51, responsible for the coordination of the two [4Fe–4S] clusters. From the comparison of its amino acid sequence with other 7Fe and 8Fe Fds (Fig. 1), it is observed that the characteristic pattern in the cysteinyl ligand region is maintained [38] and indicate that Cys11, 14, 17 and 51 are cluster I ligands, and Cys21, 41, 44 and 47 binds cluster II. Similarity is found to the already characterized 8Fe Fds from *Pa* (53% similarity), *Cp* (47% similarity), *Cau* (47% similarity) and *Cv* (27% similarity), to which a 3D structure is

already available, and also to *Dmb* (80% similarity), with a stronger analogy to this last one, what was expectable since they are both proteins from the same organism but different strains. The sequences were compared using the BIOEDIT sequence alignment editor [39]. A glutamic acid residue is present after the last cysteinyl residue (Cys 51) in the sequence of *Dd27 Fd*, replacing a well conserved proline, a typical residue in the amino acid sequence of 8Fe Fds. Another exception is observed for the 7Fe Fd from *Da* [38].

The native *Dd27Fd* was found to be a homodimer based in the molecular weight of 13.4 kDa, determined by gel filtration, and a value of with 6.4 kDa for the monomer, from the amino acid sequence. A molar absorptivity ( $\epsilon$ ) of  $28\,700\text{ M}^{-1}\text{ cm}^{-1}$  at 390 nm was calculated for the native protein, using the protein quantification assay. The number of iron atoms per monomer obtained was 7.6, confirming the presence of  $2 \times [4\text{Fe}–4\text{S}]$  clusters, or a mixture of  $[3\text{Fe}–4\text{S}] + [4\text{Fe}–4\text{S}]$  and  $2 \times [4\text{Fe}–4\text{S}]$ , as it will be discussed with the information obtained from the spectroscopic data.

### 3.1. NMR spectroscopy

We analyzed different oxidation states of the Fd: the native state, as obtained after anaerobic purification (*Dd27nat*), the reduced state (*Dd27red*) after the addition of dithionite to the native protein, and the reoxidized state (*Dd27ox*) produced after reoxidation with air of the reduced sample.

The low-temperature EPR spectra of both the *Dd27nat* and *Dd27red* (not shown) indicate the presence of [4Fe–4S] and [3Fe–4S] clusters. In the native state an anisotropic signal centered at  $g = 2.01$  was observed, characteristic of a [3Fe–4S] in the oxidized +1 state,  $S = 1/2$  [38,40]. In the reduced state a rhombic signal was observed, with  $g$  values of 2.07, 1.94 and 1.89, assigned to the [4Fe–4S] centers in the reduced state +1, with  $S = 1/2$  [41]. The 1D  $^1\text{H}$  NMR spectra of the *Dd27Fd* in the native (A, B), reduced (C) and reoxidized (D) states are presented in Fig. 2. The spectrum of *Dd27nat* Fd show resonances at low field. Resonance at approximately 26 ppm presents a chemical shift and a Curie temperature dependence typical of a  $\beta\text{-CH}_2$  proton of a cysteinyl ligand of [3Fe–4S] center [42].

<i>CauFd</i>	AYVINEACI--S	GGAGEPECPVNAI--SSGDDRYVIDADTCIDCG-----ACAGVCVPDAPVQA
<i>CvFd</i>	ALMITDQCI--NCNVGEPECPNGAI--SQGDETYVIEPFLCTECVGHYETSQCQVEVCVPVDCIKDP	
<i>PaFd</i>	AYVINDSCI--ACGACKPECPVNCI--NNGAIYVIDADSCIDCG-----SCASVCPVGAPNPED	
<i>CpFd</i>	AYKIADSCV--SCGACASECPVNAI--SQGDSIFVIDADTCIDCG-----NCANVCVPVGAPVQE	
<i>DmbFd</i>	MGYSVIVDSDKCI--GGGECVDVCPVEVYELQNGKAVPVNEEECLGCE-----SCIEVCQNAI-VE	
<i>Dd27Fd</i>	GYNVNVDVDKCI--GGGECVDVCPVEVYELKDSKAEAVSPEECCLGCE-----SC?TVCEVNAITDEN	
<i>PnFd</i>	AFIVTDNCIKCKYTDCEVCVPVDCFYEGP-NFLVI?PDECIDCA-----LCEPECFAIFSED	
<i>DaFdIII</i>	GYKITIDTDKCT--GDGECVDVCPVEVYELQDGKAVAVNEDECCLGCE-----SCVEVCEQDALTVEEN	
<i>CvFd</i>	SHEETEDELRAKYERITGEG	

Fig. 1. Amino acid sequence alignment of several Fds containing  $2 \times [4\text{Fe}–4\text{S}]$  or  $[3\text{Fe}–4\text{S}] + [4\text{Fe}–4\text{S}]$  clusters. The cysteinyl cluster ligands are marked in gray: the firsts three cysteines (or two, in 7Fe Fds) and the last one in the sequence binds cluster I; the other four binds cluster II. 8Fe Fds: *Cau*, *Clostridium acidurici* [51], *Cv*, *Chromatium vinosum* [46], *Pa*, *P. aerogenes* [26], *Cp*, *C. pasteurianum* [26], *Dmb*, *D. baculatum* Norway 4 [25], *Dd27*, *D. desulfuricans* ATTC27774. 7Fe Fds: *Pn*, *P. nautical* [50], *Da*, *Desulfovibrio africanus* [27].

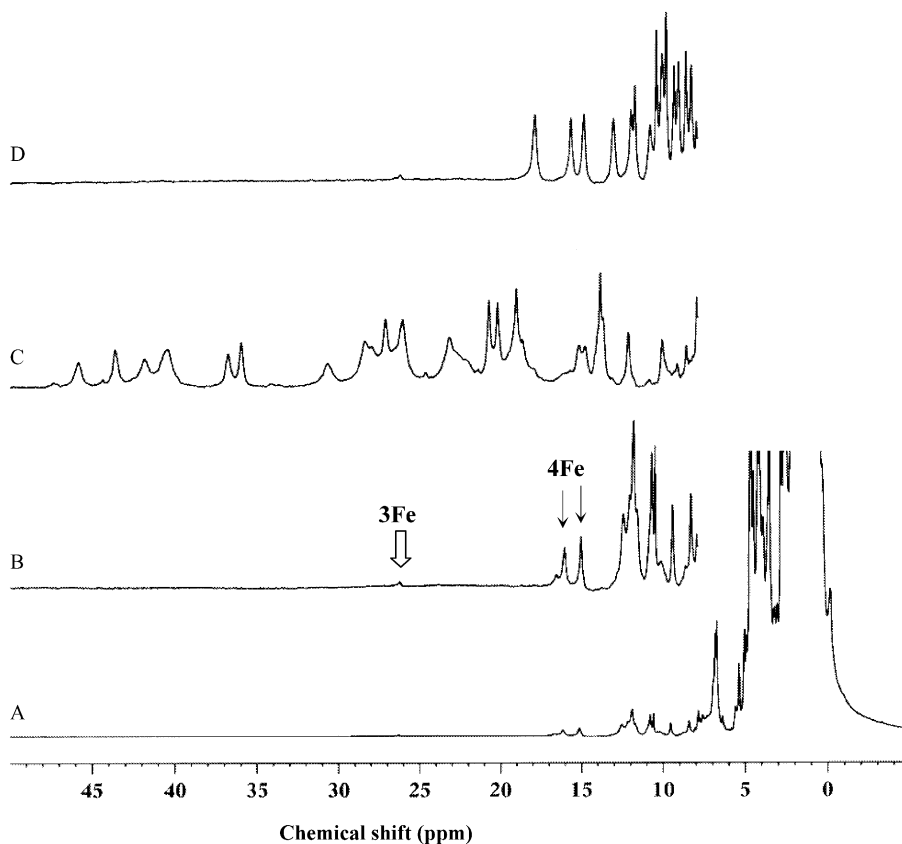


Fig. 2. 1D  $^1\text{H}$  NMR spectra at 400 MHz of *Dd27Fd* (with water peak suppression). (A) Full spectrum of the anaerobically purified protein (*Dd27nat* Fd); (B) expanded low field region of *Dd27nat* Fd; (C) dithionite reduced state (*Dd27red* Fd) and (D) reoxidized state (*Dd27ox* Fd).

The two resonances in the 16 ppm region (labeled 4Fe in Fig. 2(B)) are characteristic of  $\beta\text{-CH}_2$  protons of cysteinyl ligands of [4Fe–4S] centers in the oxidized state and they show anti-Curie temperature dependences. The area ratios of these resonances suggest that the 3Fe cluster is present as 25%, compared with 4Fe centers. These assignments are supported the analogy with the NMR spectra and the temperature dependences of the 7Fe Fd from *Desulfurolobus ambivalens* [43] and Fds I and II from the sulfate reducer *D. gigas* (*Dg*) that contain one [4Fe–4S] and one [3Fe–4S] centers, respectively [42,44]. In agreement, the EPR measurements indicate that the amount of 3Fe centers is substoichiometric.

The 1D  $^1\text{H}$  NMR spectrum of *Dd27red* Fd show resonances shifted to lower field, indicating an increase of paramagnetism due to reduced [4Fe–4S] centers with  $S = 1/2$  (Fig. 2(C)). The temperature dependence of these resonances (not shown) revealed a mixture of Curie and anti-Curie behavior, characteristic of proteins containing one or two [4Fe–4S] clusters in the reduced state [42,45,46]. This state is not stable for a long period of time, in the experimental working NMR conditions and tends to return to an oxidized state.

After reoxidation (Fig. 2(D)) the low field region of the 1D  $^1\text{H}$  NMR spectrum of *Dd27ox* Fd shows the

presence of [3Fe–4S] centers (resonance at ca. 26 ppm) but with lower intensity compared with the native sample, indicating that redox cycling rebuilds 4Fe cores. The set of well-defined resonances in the spectral region between 7 and 20 ppm is quite homologous to those presented by other 8Fe Fds in the oxidized state, as compared with *Cau*Fd [45] and *Cp*Fd [46]. The temperature dependence followed by these resonances has anti-Curie type behavior (except for resonance at 26 ppm) (Fig. 3).

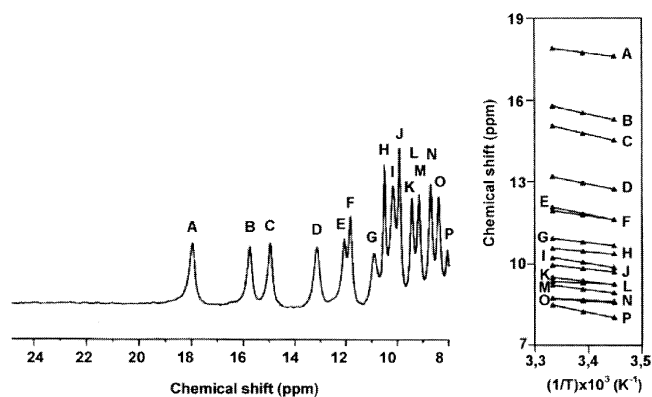


Fig. 3. Low field region of the 1D  $^1\text{H}$  NMR spectrum, at 400 MHz, of *Dd27ox* Fd, and temperature behavior of resonances labeled from A to P.

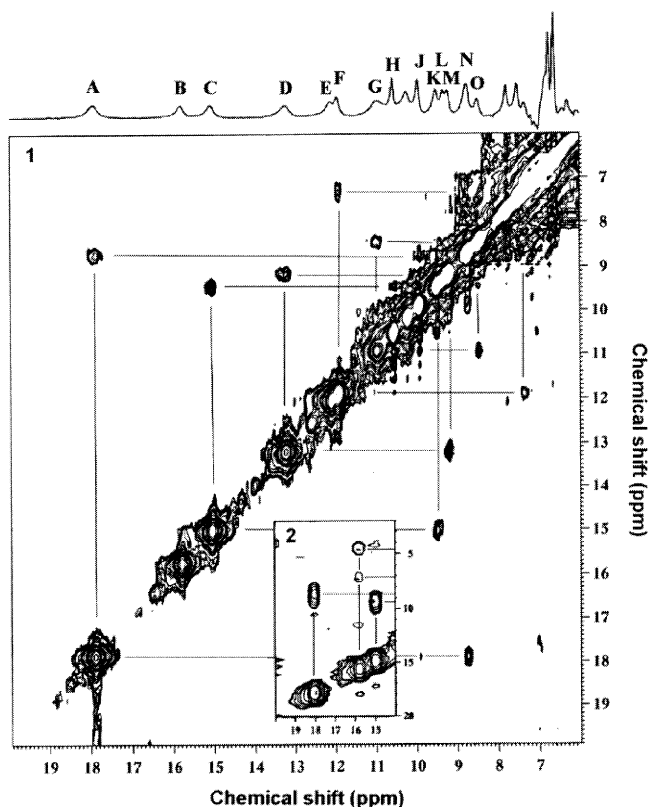


Fig. 4. 2D  $^1\text{H}$  NOESY NMR spectra at 400 MHz, from lower field region of *Dd27ox* Fd, in  $^2\text{H}_2\text{O}$  (pH 7.6), 300 K. Resonances are labeled A to O. (1) 5 ms mixing time; (2) 10 ms mixing time.

### 3.2. Assignment of the iron–sulfur cluster cysteinyl ligand protons of *Dd27ox* Fd

The NOESY spectra of *Dd27ox* Fd, acquired with two different mixing times, are shown in Fig. 4. These spectra allow the assignment of seven of the eight  $\beta\text{-CH}_2$  protons of the cysteinyl ligands that coordinate the two

$[\text{4Fe-4S}]$  clusters, as well as some of the  $\alpha\text{-CH}$  protons (Table 1). The anti-Curie temperature dependence and the fast relaxation times detected allowed to assign the resonances of *Dd27ox* Fd to the  $\beta\text{-CH}_2$  and  $\alpha\text{-CH}$  protons of the cysteines that coordinate the clusters. Resonances A, N and J are correlated. Considering cross peak intensities, the  $T_1$  values and the temperature dependence behavior, it is possible to assign both  $\text{H}_\beta$  and  $\text{H}_\alpha$  from the same cysteinyl residue. Resonance B is correlated with two other resonances at 4.8 and 7.5 ppm, assigned to protons  $\text{H}_\beta$  and  $\text{H}_\alpha$ , respectively. It is also possible to observe correlations between resonances C and K, D and M, E and 7.8 ppm, F and 7.3 ppm and G and O, all assigned to the pair of  $\text{H}_\beta$  protons from each of the coordinating cysteinyl residues. To resonances H and I it was not possible to observe any correlation.

The specific assignment of these resonances to the  $\alpha\text{-CH}$  and  $\beta\text{-CH}_2$  protons of the cysteines that coordinate the Cluster I and II was made, considering the homology between the polypeptide chain of *Dd27Fd*, *DmbFd*, *CpFd* and *PaFd* (Fig. 1), the solution structure of *CpFd* [47] and X-ray structure of *PaFd* [48] and the homology between the 3D structures of *PaFd* and *DmbFd* [25]. 1D and 2D NOESY  $^1\text{H}$  NMR information published for *CpFd* was also used. All the information is gathered together in Table 1.

### 3.3. Angular dependence of the paramagnetic resonances

NMR studies on  $[\text{4Fe-4S}]^{+3}$  model compounds and in proteins containing  $[\text{4Fe-4S}]^{+2}$  clusters, established that the isotropic interaction of the protons situated at the closest carbon to the iron atom is a function of the dihedral angle  $\theta$ , defined by the four atoms  $\text{H-C-S-Fe}$  [45,49]. Using the equation  $\delta = a \sin^2(\theta) + b \cos(\theta) + c$ , a graphic representation of the paramagnetic shifts from

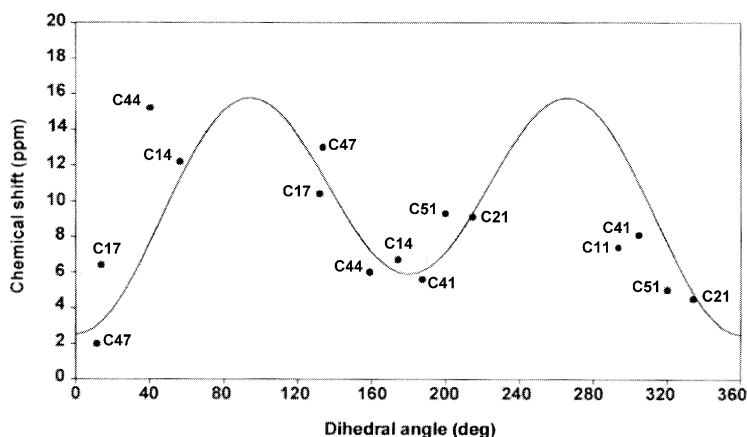


Fig. 5. Best fitting for equation  $\delta = a \sin^2(\theta) + b \cos(\theta) + c$  ( $a = 12.2$ ,  $b = -3.9$  and  $c = 3.232$ ) that relates the hyperfine chemical shifts of the  $\beta\text{-CH}_2$  protons of the coordinate cysteinyl residues of *CauFd* obtained by NMR, with the corresponding dihedral angles  $\text{Fe-S}_\gamma\text{-C}_\beta\text{-H}_\beta$  from *CauFd*, obtained from its X-ray structure. The  $\beta\text{-CH}_2$  protons of the coordinate cysteinyl residues of *Dd27Fd*ox are indicated, excluding the  $\beta\text{-CH}_2$  protons of Cys11.



Table 1

Spectral NMR parameters at 300 K, pH 8.0 and specific assignment of the resonances of the  $\beta$ -CH<sub>2</sub> and  $\alpha$ -CH protons that coordinate the [4Fe–4S] clusters I and II in *Dd27ox*

Resonance	Protons	$\delta$ (ppm)	$T_1$ (ms)	Assignment	
				Cluster	Specific assignment
A	$\beta$ -CH	18.0	3.5	II	Cys 44
N	$\beta$ -CH	8.8	9.6		
J	$\alpha$ -CH	10.0	14.0		
B	$\beta$ -CH	15.8	4.5	II	Cys 47
	$\beta$ -CH	4.8	n.d		
	$\alpha$ -CH	7.5	n.d		
C	$\beta$ -CH	15.0	4.3	I	Cys 14
K	$\beta$ -CH	9.5	9.8		
D	$\beta$ -CH	13.2	4.8		
M	$\beta$ -CH	9.2	8.9	I	Cys 17
	$\beta$ -CH	12.1	5.2		
	$\beta$ -CH	7.8	n.d		
F	$\beta$ -CH	11.9	7.8	II	Cys 21
	$\beta$ -CH	7.3	n.d		
	$\beta$ -CH	10.9	3.2		
G	$\beta$ -CH	10.9	3.2	II	Cys 41
O	$\beta$ -CH	8.4	9.7		
I	$\beta$ -CH	10.2	5.5		
H	$\alpha$ -CH	10.5	22.1	I	Cys 11

n.d., non determined.

the  $\beta$ -CH<sub>2</sub> protons of the cysteinyl residues of *CauFd*<sub>ox</sub>, versus the dihedral angles obtain from its X-ray structure was done [45]. The  $\beta$ -CH<sub>2</sub> protons of the coordinate cysteinyl residues of *Dd27Fd*<sub>ox</sub> are indicated, with exception for one of the  $\beta$ -CH<sub>2</sub> protons of cysteine 11, to which the chemical shift is unknown. The best fitting for the equation originated values of **a** = 12.2, **b** = −3.9 and **c** = 3.232 (Fig. 5). A positive **a** value means that the spin delocalization mechanism is essentially  $\pi$ , however, the **b** value different from zero suggests that the spin delocalization mechanism should not be neglected [49]. Assuming the same  $\theta$  values for *Dd27ox* Fd and *CauFd*<sub>ox</sub>, the chemical shifts of the  $\beta$ -cysteinyl protons of *Dd27ox* Fd can be analyzed, after subtracting 2.8 ppm for the diamagnetic contribution. A good agreement is observed for all the protons with a slightly deviation for one of the signals of Cys44 (and Cys17). A similar deviation has been already observed for the correspondent Cys40 in *CvFd* [41]. This disturbance seems to arise, not from a global change in the electronic structure of the [4Fe–4S]<sup>+2</sup> cluster, but instead from a local one in neighborhood of cysteine 40, since all the other chemical shifts associated with the other ligands of the same aggregate maintain its values practically unchanged.

The Fd isolated from *D. desulfuricans* ATCC 27774 is sensitive to oxygen. The amino acid sequence contains cysteines, in number and position, capable to accommodate 2 × [4Fe–4S] clusters. The cluster instability results in cluster damaged. This fact made the analysis of the NMR data more complex due to the presence of [3Fe–4S] clusters. It was also shown that cluster

interconversion may occur under reducing conditions. The proton cysteinyl resonances due to the oxidized tetranuclear sites show remarkable differences when the native anaerobic purified preparation (*Dd27nat*) and the reoxidized sample (*Dd27ox*) are compared. An identical observation has also been documented for *DgFd* I, a protein that contains one [4Fe–4S], where the spectrum of the native form purified under aerobic conditions, differs from the spectrum of the same protein purified in the absence of oxygen [52]. Moreover, in *Dd27* Fd, the replacement of the strategic proline by a glutamic residue, in cluster II proximity, may have consequences in subtle variations of dihedral angles, which may reflect in cluster spin delocalization.

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